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Positive- and negative-acting signals combine to determine differential RNA replication from the paramyxovirus simian virus 5 genomic and antigenomic promoters

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Abstract

The *cis*-acting signals found at the 3′ ends of the genomic and antigenomic RNAs are a major factor determining the level of paramyxovirus RNA replication from each promoter. Using a minigenome system that reconstitutes SV5 RNA synthesis from cDNA-derived components, we show here that the genomic promoter (GP) for the paramyxovirus SV5 directs RNA replication ~14-fold lower than that seen from the antigenomic promoter (AGP). The goal of this study was to identify *cis*-acting signals responsible for differential levels of RNA replication from the SV5 GP and AGP. We have previously shown that the SV5 AGP contains three sequence-dependent elements (CRI, CRII, and Region III) that are separated by sequence-independent spacer regions. Minigenomes containing chimeric promoters were constructed to test the hypothesis that transfer of discrete *cis*-acting AGP elements to the GP could confer higher replication properties to the GP. Minigenomes containing a substitution of the AGP CRI, CRII, or Region III elements alone in place of the corresponding GP sequences did not show enhanced levels of RNA replication. However, transfer of both the AGP 3′ terminal CRI and Region III elements into the corresponding sites of the GP led to a minigenome which replicated to ~40% of the levels seen with the AGP. This enhanced RNA replication from the GP was further increased up to AGP levels by also including the intervening AGP segment (bases 20–50) located between CRI and Region III. Importantly, transfer of nonviral sequences in place of GP bases 20–50 also increased RNA replication to levels approaching that of the AGP, but only in the context of the AGP CRI and Region III substitutions. These data indicate that differential levels of RNA replication from the SV5 GP and AGP are due to a combination of positive-acting signals in the AGP (CRI and Region III) and a negative-acting signal in the GP (bases 20–50). Possible functions for the SV5 promoter elements in determining RNA replication levels are proposed.

Introduction

The paramyxovirus genomic and antigenomic RNAs contain 3′ end *cis*-acting signals that are a major factor controlling viral RNA replication (Calain and Roux, 1993; Fearns et al., 2002; Hoffman and Banerjee, 2000b; Keller et al., 2001; Taparrel and Roux, 1996). Typically, viral genomes are found at high levels compared to antigenomic RNA (Kolakofsky and Bruschi, 1975; Le Mercier et al., 2002; Mottet and Roux, 1989). These genomic RNAs serve at least three functions in the growth cycle: incorporation into progeny virions, acting as a template for producing mRNAs, and acting as a template for antigenome synthesis (Lamb and Kolakofsky, 2001). By contrast, antigenomic RNA is typically found in lower amounts and apparently functions only as a template to amplify genomic RNA. Thus, paramyxovirus RNA replication directed by the genomic promoter (GP) to produce antigenomes is typically much lower than that from the antigenomic (AGP) which produces genomes. In this report, we have identified *cis*-acting signals in the GP and AGP that are responsible for differential RNA replication for the prototypic paramyxovirus simian virus 5 (SV5).

Paramyxoviruses are a diverse family of enveloped viruses whose genome is composed of a single strand of

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negative-sense RNA (Lamb and Kolokofsky, 2001). Prototypic paramyxoviruses that have been extensively studied include Sendai virus (SeV), measles virus, and respiratory syncytial virus (RSV). These viruses are members of the respiro-, morbilli-, and pneumoviruses, respectively. SV5 is the prototype of the rubulaviruses, which also includes mumps virus, SV41, and human parainfluenza virus type 2 (HPIV2).

While paramyxoviruses share many general features of viral RNA replication, reverse genetics systems (reviewed in Conzelmann, 1996; Nagai, 1999; Palese et al., 1996) have uncovered significant differences in requirements for *cis*-acting sequences in the genomic and antigenomic RNAs (Durbin et al., 1997; Fearns et al., 2002; Hoffman and Banerjee, 2000a; Keller et al., 2001; Murphy et al., 1998; Taparrel and Roux, 1996). For example, the overall length of a paramyxovirus RNA template can be a major factor determining the level of RNA replication, with genome replication being most efficient when the total number of nucleotides is an even multiple of six (Calain and Roux, 1993; Kolakofsky et al., 1998). Recent results from the analysis of SeV minigenomes supports a model for the "rule-of-six" constraint in which the viral promoter sequences must be recognized in the context of NP monomers, each of which binds six nucleotides (Egelman et al., 1989). The degree to which replication of a particular paramyxovirus genome adheres to the rule-of-six requirement differs among viruses. For SeV, the rule of six is an apparent strict requirement for RNA replication (Calain and Roux, 1993), although expression of the SeV C protein can have a profound effect on the stringency for 6N length genomes (Tapparel et al., 1997). By contrast, there is no replicative advantage to RSV genome analogs having 6 N-length genomes (Samal and Collins, 1996). For SV5 and HPIV3, the rule-of-six requirement is intermediate between the stringencies found previously for SeV and RSV (Murphy and Parks, 1997; Durbin et al., 1997).

The promoters for paramyxovirus RNA replication are major determinants of the level of RNA replication and are located at the 3′ ends of the genomic and antigenomic RNAs (reviewed in Lamb and Kolakofsky, 2001). For SV5, previous mutational analyses have identified three discontinuous sequence-dependent elements required for optimal RNA replication from the AGP (Keller et al., 2001; Murphy et al., 1998; Murphy and Parks, 1999). Conserved Region I (CRI) is composed of the 3′ terminal 19 nucleotides and was identified through a sequence alignment as a region that is highly conserved between rubulavirus antigenomes (Fig. 1). Conserved Region II (CRII) was identified by mutational analysis as an internal promoter element within the coding region of the L protein gene located 73 to 90 from the 3′ end of the antigenomic RNA (Fig. 1; Murphy and Parks, 1998). Extensive mutagenesis (Murphy and Parks, 1999) has defined an important motif in the SV5 AGP CRII as a 3′-GC-5′ dinucleotide pair located in the first two positions of three sequential hexamers of nucleotides (template strand, 3′-AGGAGCGGUAGCUAGGGC-5′; Fig. 1). SeV genomic and antigenomic RNAs also contain an internal promoter element corresponding to the SV5 CRII (Taparrel and Roux, 1998). Remarkably, the SeV internal element differs from SV5 in both location (bases 79–96) as well as sequence composition (template strand, 3′-CNNNNN-5′). For the SV5 AGP, a third promoter element (bases 51–66; boxed Region III; Fig. 1) has been identified that is not essential for RNA replication, but appears to act as an enhancer.

The goal of the experiments described here was to define the *cis*-acting elements responsible for differential levels of RNA replication directed by the SV5 GP and AGP. A sequence alignment of the 90 bases at the 3′ end of the genomic and antigenomic RNA shows that while the GP contains CRI and CRII elements, the equivalent of the AGP Region III is occupied in the GP by a segment encoding the NP transcriptional start site (Fig. 1). We have tested the hypothesis that the SV5 GP lacks positive-acting signals for high levels of RNA replication and that transfer of discrete elements (i.e., CRI, CRII, or Region III) from the AGP to the GP could confer higher RNA replication properties to the GP. Our results indicate that differential RNA replication from the SV5 GP and AGP is due to a combination of positive-acting sequences in the AGP and also a negative-acting segment in the GP. Possible functions for the SV5 promoter elements in determining RNA replication levels are discussed.

Results

The genomic 3′ terminal 90 bases direct optimal RNA transcription and replication

Previous work has shown that the 3′ terminal 90 bases of the SV5 AGP are the minimal sequence that can direct efficient RNA replication (Murphy and Parks, 1997), and this has been confirmed through mutational analyses (Murphy et al., 1998). However, the minimal sequences required for the SV5 GP to direct optimal RNA synthesis had not been determined.

A minigenome (90GP-GL) was constructed that contained a reporter gene GL flanked on the 3′ end by the terminal 90 bases of genomic RNA and on the 5′ end by 113 bases of 5′ L-trailer sequences (Fig. 2A). RNA synthesis from the 90GP-GL minigenome was compared to a previously described minigenome AGP-GL (Murphy et al., 1998), which differs only by having 90 bases of antigenomic RNA, instead of genomic RNA, at the 3′ end. RNA synthesis directed by these two minigenomes was assayed using a previously described transfection system that reconstitutes RNA synthesis from cDNA-derived components (Murphy et al., 1998; Rassa and Parks, 1999). Briefly, A549 cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vacT7; Fuerst et al., 1986)

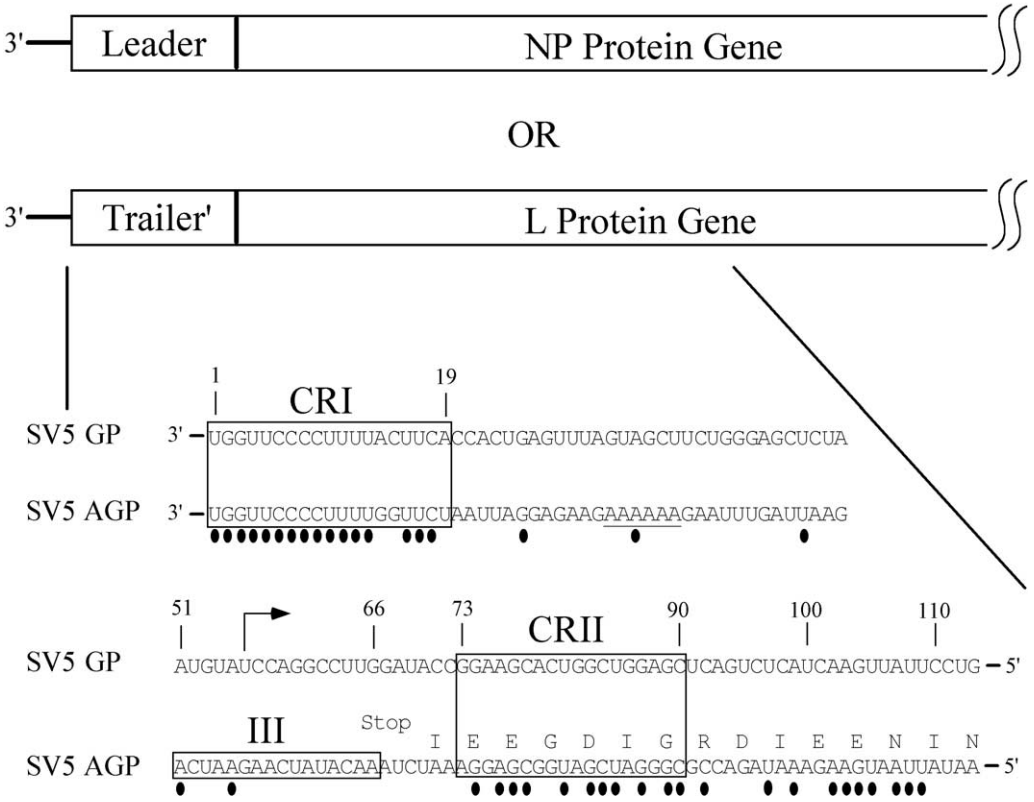

Fig. 1. Sequence comparison of the SV5 GP and AGP. The 3′-terminal 113 bases from the genomic and antigenomic RNA of SV5 are listed. Ninety bases of 3′ genomic RNA are designated as SV5 GP (GP) and the 90 bases of 3′ antigenomic RNA are designated as SV5 AGP (AGP). The arrow at base 56 of the GP denotes the transcriptional start site of the NP gene. Amino acids that are encoded in the 3′ end of the SV5 L gene are designated by one-letter abbreviations above the AGP sequence. The site for the poly(A) addition to the L mRNA is underlined. Conserved Region I (CRI; 3′ terminal), conserved Region II (CRII; within the NP or L gene, for the genomic or AGP, respectively), Region III are indicated by boxes. Filled circles denote positions of sequence identity.

and then transfected with plasmids expressing the SV5 NP, P, and L proteins. The transfection mix also included a plasmid expressing a negative-sense RNA corresponding to the minigenome 90GP-GL or AGP-GL. To detect products of SV5 RNA replication, cell lysates were treated with micrococcal nuclease to digest RNAs not encapsidated by NP (Peeples and Collins, 2000), and RNA was extracted. RNA transcription products were isolated from parallel samples using oligo(dT) cellulose (Rassa and Parks, 1999). To detect RNAs synthesized by the L polymerase, rather than the T7 polymerase, RNA samples were analyzed by Northern blotting with a GL-specific riboprobe that was the same polarity as the cDNA-derived T7 transcript. As shown in Fig. 2B, the 90-base GP directed RNA replication to ~7% that of the WT AGP (compare lanes 1 and 3). As expected, the GP directed the synthesis of a polyadenylated transcription product, while the AGP could not direct transcription (Fig. 2B, lanes 1 and 3, transcription panel). These data indicate that the GP is much weaker than the AGP in its ability to direct RNA replication and that the GP directs predominantly mRNA transcription rather than replication products.

The alignment in Fig. 1 also shows a region located 5′ to CRII that has a relatively high degree of sequence identity, with bases 97–109 being identical in 9 of 13 bases. While previous results have shown this region is not required for optimal replication from the SV5 AGP (Murphy et al., 1998), the role of this conserved region in RNA synthesis from the SV5 GP has not been determined. This raises the possibility that additional sequences are required.

To determine if additional sequences past base 90 contributed to differences in RNA synthesis from the GP and AGP, a minigenome (180GP-GL, Fig. 2B) was constructed to contain additional 3′ end sequences that extended up to the NP translational start codon at base 180 (Parks et al., 1992). When analyzed in the vacT7 transfection assay, levels of RNA replication as well as transcription from the 180GP minigenome closely matched that seen with the minigenome containing the 90-base GP (Fig. 2B, compare lanes 3 and 5). Thus, the sequences necessary for optimal RNA replication and transcription are contained within the 3′ terminal 90 bases of genomic RNA. To determine if decreasing the size of the GP altered RNA synthesis, the minigenome 60GP-GL was constructed to contained only the first 60 bases of 3′ genomic RNA, but not CRII found from bases 73–90 (Fig. 2B). As shown in Fig. 2B, the

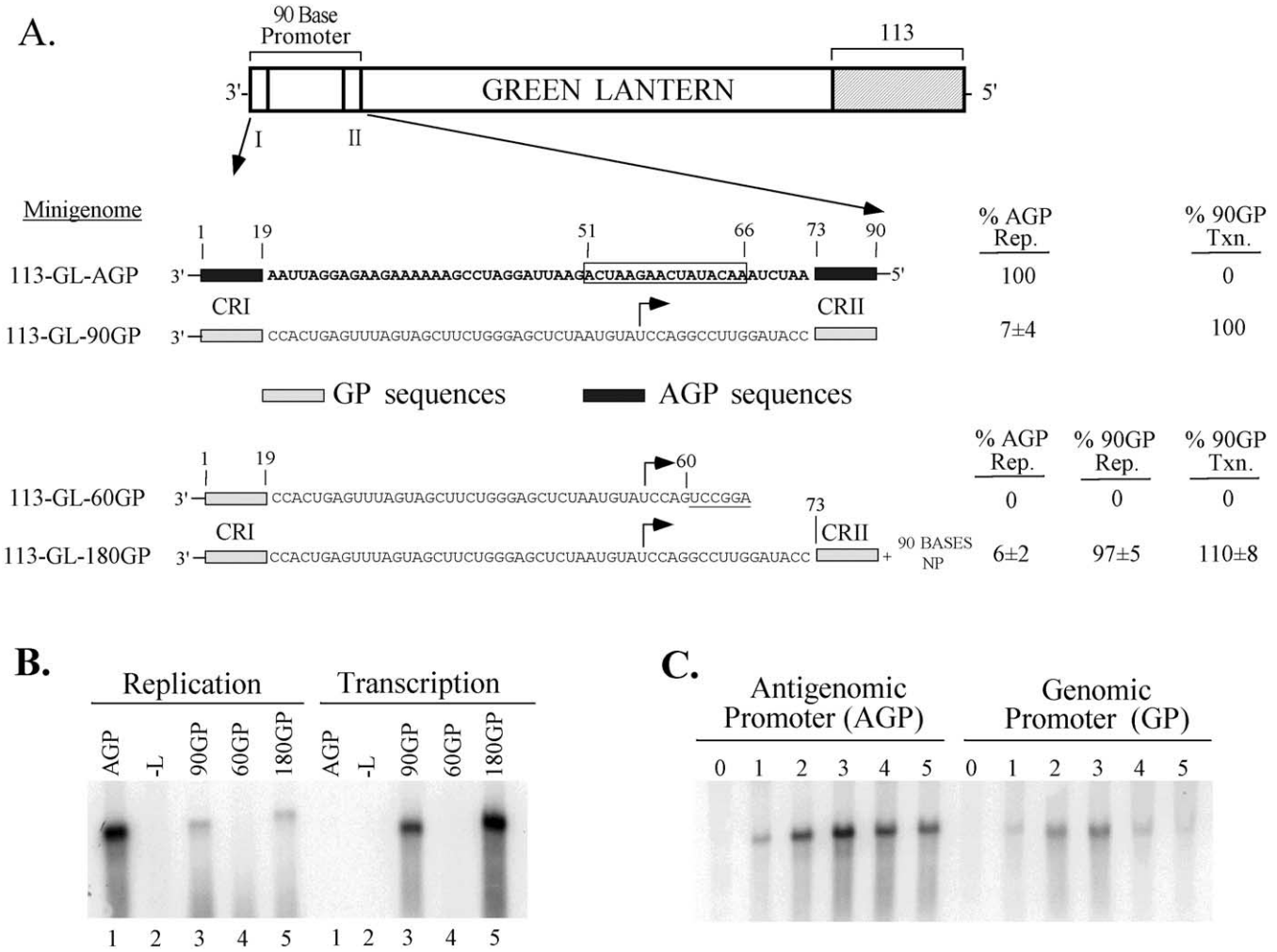

Fig. 2. The 3′ terminal 90 bases of SV5 genomic RNA directs optimal levels of RNA synthesis. (A) Schematic of the minigenomes used in the in vivo replication/transcription assay. The Green Lantern gene (GL) is flanked on the 3′ end by the 90-base GP or AGP with CRI and CRII designated by I and II and on the 5′ end by 113 bases of the L/trailer sequence (striped box). The sequences between CRI and CRII are shown for the copyback (113-GL-AGP) or internal deletion (113-GL-90GP) minigenomes and for minigenomes containing deletions or extensions of the GP (113-GL-60GP or 113-GL-180GP, respectively). CRI and CRII are shown as boxes, but have different sequences (see Fig. 1). Region III found from bases 51–66 of the AGP is boxed. The NP transcription start site (arrow at base 56) is only found in the GP. 113-GL-60GP and 113-GL-180GP have a GP containing the first 60 bases of 3′ genomic RNA or the 90-base GP along with 90 bases extending to the translational start site of NP, respectively. Underlined sequence for 113-GL-60GP denotes the *Stu*I restriction site linking the truncated promoter to GL. Relative replication and transcription for each template are expressed as a percentage of the level determined for the WT template analyzed in parallel (average ± SD). (B) A representative Northern blot for levels of replication and transcription from minigenomes containing WT and mutant GP. Lane -L, transfected cells in which the L plasmid was omitted. (C) Effect of amount of transfected NP plasmid on RNA replication from 113-GL-AGP or 113-GL-90GP.

signals surrounding the NP transcriptional start site in the truncated minigenome differ by 3 bases from the WT start site because of the linkage with the GL segment, but still closely resemble the consensus site previously published (Rassa and Parks, 1999). When assayed in the vacT7 system, no viral RNA transcription or replication products from the 113-GL-60GP minigenome were detected (Fig. 2B lane 4). Although these data indicate that CRII is required for both RNA replication and RNA transcription from the GP, the minimum sequences between bases 60 and 90 that are needed for a functional GP have not been further defined.

The above results support the proposal that the SV5 GP is inherently a weaker promoter for RNA replication than the AGP, since the GP directs ~14-fold lower RNA replication. Titration experiments were carried out to determine if RNA replication from the GP could approach or exceed that seen from the AGP by increasing the levels of NP. Cells were transfected with a constant amount of plasmids expressing L, P, and a minigenome containing either the wild-type (WT) GP or the WT AGP, along with increasing amounts of the plasmid expressing NP. RNA replication products were isolated and analyzed by Northern blotting. As shown in Fig. 2C, RNA replication from the AGP showed a broad titration with an optimal level seen using 3 μg of transfected NP plasmid. RNA replication from the GP

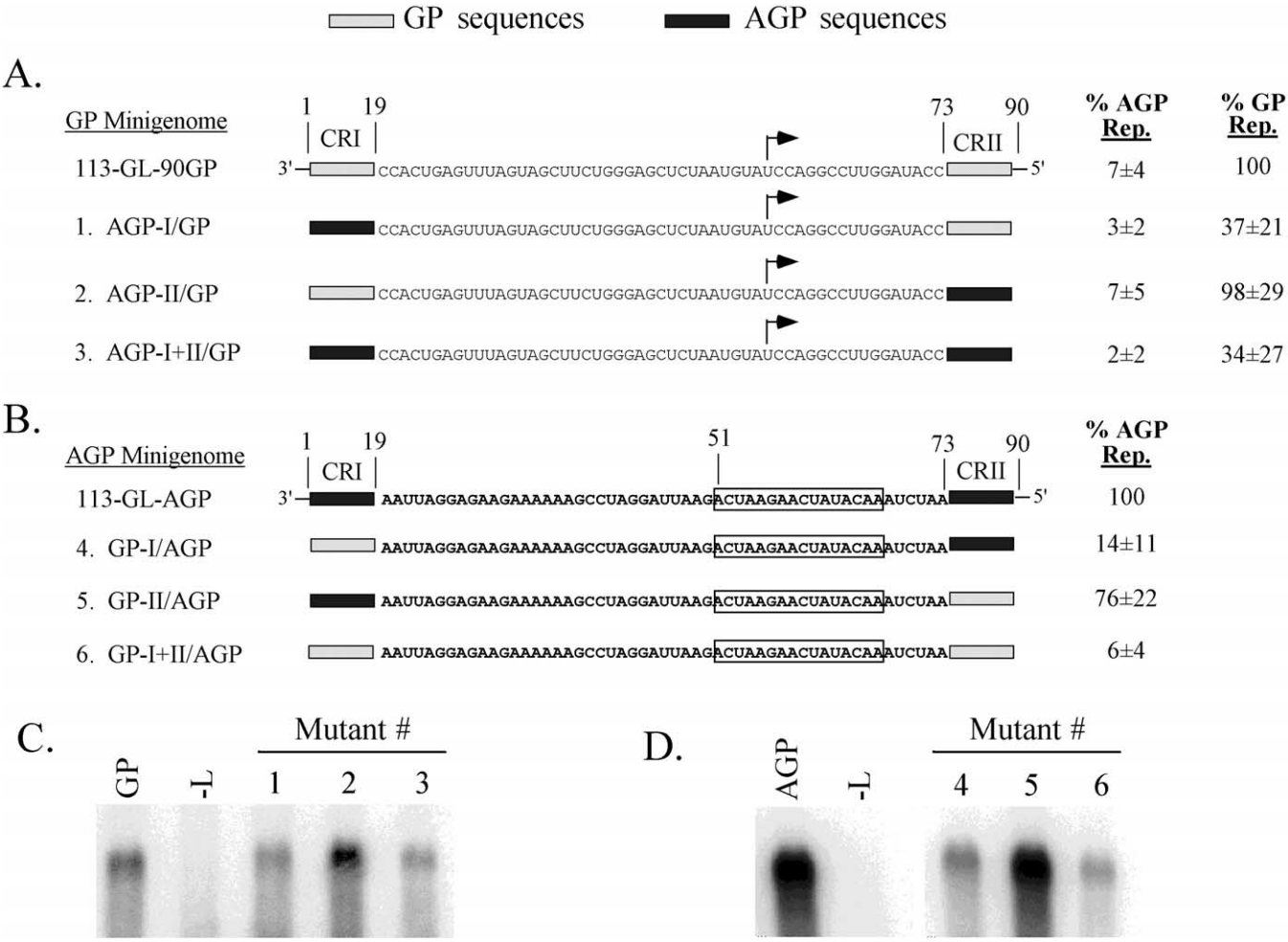

Fig. 3. RNA replication is altered in minigenomes containing exchanges of the GP and AGP CRI and CRII sequences. (A) Substitutions into the SV5 GP. The sequence of the intervening region between the GP CRI and CRII is shown with the NP transcription start site at base 56. 113-GL-90GP is the wild-type genomic promoter. Mutants 1–3 contain substitutions of CRI, CRII, or CRI+CRII from the AGP, represented as black boxes, in place of the corresponding elements in the GP, represented as stippled boxes. The replication levels of Mutants 1–3 are expressed as percentages of the levels determined for the WT GP and AGP minigenomes analyzed in parallel (average ± SD). (B) Substitution into the SV5 AGP. The sequence of the intervening region between the AGP CRI and CRII is shown with Region III found from bases 51–66 boxed. 113-GL-AGP is the wild-type antigenomic promoter. Mutants 4–6 contain exchanges of CRI, CRII, or CRI+CRII from the GP, represented as stippled boxes, in place of the corresponding elements in the AGP, represented as black boxes. The replication levels of Mutants 4–6 are expressed as percentages of the level determined for the WT GP minigenome (average ± SD). Representative Northern blots of replication products from Mutants 1–3 (C) or 4–6 (D) are shown. Lane -L, transfected cells in which the L plasmid was omitted.

was also optimal using 3 μg transfected NP plasmid, but maximal replication levels were always much lower than those seen with the AGP. The ratio of RNA replication to transcription from the GP was not significantly altered by transfecting increased levels of NP plasmid (not shown). Taken together, these results indicate that the SV5 GP is inherently weaker than the AGP in its ability to direct RNA replication.

High-level RNA replication can be altered by exchanges of AGP and GP CRI, but not CRII, sequences

As shown in Fig. 1, the SV5 GP has sequences corresponding to the AGP CRI and CRII. Based on this high sequence identity, we hypothesized that CRI and CRII elements within the AGP and GP could be interchanged without affecting RNA replication. To test this hypothesis, minigenome Mutants 1–3 were constructed such that the 3′ GP (Fig. 3A, gray boxes) contained an exchange of the AGP CRI alone, CRII alone, or CRI + CRII (black boxes). For example, Mutant 1 contains the AGP CRI sequences (black box) substituted in place of the corresponding 3′ end segment in the GP to give the AGP-I/GP chimera (AGP CRI transferred into the 3′ end GP). RNA replication levels from Mutants 1–3 were analyzed by Northern blotting and expressed as a percentage of the WT GP as well as WT AGP (Fig. 3A and C). When the AGP CRI (Mutant 1) or CRI + CRII (Mutant 3) was substituted into the GP, RNA replica-

tion was slightly decreased to ~37 and ~34% of WT GP levels, respectively (Fig. 3A and C). Based on variability in our minigenome assay, we have defined significant changes in RNA synthesis as being increased fourfold or decreased to 25% of WT levels (Rassa and Parks, 1998). Substituting the AGP CRII into the GP did not significantly alter GP activity, and replication was ~98% that of the WT GP (Fig. 3C, Mutant 2; quantitated in Fig. 3A). Very similar results were seen with transcription products from the mutant promoters (data not shown). These data indicate that the CRII element from the AGP can replace the CRII element in the GP without significantly altering RNA replication. Surprisingly, replacing the GP CRI with the AGP CRI resulted in a decrease in RNA replication when exchanged individually or with the AGP CRII (Fig. 3A and C). However, when RNA replication is expressed as a percentage of WT AGP, the differences in low-level replication seen between Mutant 1 and Mutant 3 and WT GP are not as evident.

In the converse experiment to that in Fig. 3A, a second set of minigenomes was constructed such that the 3′ AGP contained an exchange with the GP CRI alone, CRII alone, or CRI + CRII (Fig. 3B, Mutants 4–6). Analysis of RNA replication levels from these mutants showed that when the GP CRI was transferred alone (Mutant 4) or with CRII (Mutant 6) RNA replication levels were significantly reduced to ~14 and ~6% of WT AGP levels, respectively (Fig. 3D; quantitated in Fig. 3B). By contrast, transferring CRII from the GP to the AGP had little effect on RNA replication (Fig. 3D, Mutant 5; quantitated in Fig. 3B). Two important conclusions can be drawn from these chimeric minigenomes. First, CRII can be exchanged between promoters without significantly affecting RNA replication. Second, CRI exchanges, alone or in combination with CRII, decreased RNA replication from the chimeric promoters (Fig. 3). These data indicate that CRI is not interchangeable between the two promoters and is a negative-acting signal for RNA replication when it is not in the proper context of each promoter (i.e., AGP CRI with AGP; GP CRI with GP).

Identification of RNA elements from the SV5 AGP that can confer higher RNA replication to the GP

The sequence alignment of the SV5 promoters (Fig. 1) indicates that the GP does not contain the Region III enhancer found from bases 51–66 of the AGP. To determine whether transferring only AGP Region III to the GP could confer higher RNA replication to the GP, a minigenome (113-GL-AGP-III/GP; Fig. 4A) was constructed such that bases 51–66 of the GP were replaced with Region III from the AGP. When analyzed in the vacT7 system, the Mutant 7 113-GL-AGP-III/GP minigenome did not direct the synthesis of poly(A) transcription products (Fig. 4B, compare lanes 3 and 4, transcription panel), a result that is consistent with the removal of the NP transcriptional start site due to replacing bases 51–66 of the GP with Region III. More importantly, the addition of Region III to the GP did not result in a minigenome with increased RNA replication above levels seen with the WT GP (Fig. 4B, compare lanes 3 and 4, replication panel). Thus, while Region III acts to promote RNA replication from the AGP (Keller et al., 2001), it cannot by itself confer enhanced RNA replication to the GP.

To determine which AGP elements confer increased RNA replication to a GP, the AGP-III/GP minigenome, which does not direct transcription but directs RNA replication to WT GP levels, was used as a backbone to make exchanges with additional regions from the AGP. A series of chimeric minigenomes containing Region III were constructed such that the GP CRI, CRII, or CRI + CRII was replaced by the corresponding RNA elements from the AGP (Fig. 4A, Mutants 8–10). For example, Mutant 8 contains the AGP CRI (black box) and Region III (boxed sequences) substituted into the GP to create the AGP-I+III/GP chimera. Minigenome templates were analyzed for levels of RNA replication using the vacT7 assay and are expressed as a percentage of the replication levels from the WT AGP (Fig. 4A, Mutants 8–10). Transfer of the AGP CRI and Region III (Mutant 8) gave a reproducible increase in the level of RNA replication to ~40% of the AGP control (Fig. 4B, compare Mutant 8 and AGP lanes). By contrast, transfer of the AGP CRII plus Region III (Mutant 9) did not significantly increase promoter activity, and the ~7% levels of RNA replication were similar to a GP containing Region III alone (Mutant 7). Together with the results in Fig. 3 above showing that transfer of the AGP CRI alone did not confer higher replication to the GP, these data indicate that CRI and Region III can promote RNA replication from the GP, but only when present together. Unexpectedly, transfer of all three AGP elements to the GP resulted in a minigenome (Fig. 4C, Mutant 10) that directed RNA replication to levels that were not above that seen with the WT GP.

Bases 1–66 of the SV5 GP contain a negative-acting sequence that inhibits RNA replication

The above results indicated that the transfer of the AGP CRI and Region III together in place of the corresponding GP sequences increased RNA replication above that seen with the WT GP (Mutant 8), but the levels were only ~40% of WT AGP levels. Based on this result, we hypothesized that the region between CRI and Region III of the AGP might contain additional sequences that could further promote RNA replication, perhaps linking signals in CRI and Region III. To test this, a minigenome was constructed such that the 3′ end promoter was composed of the first 66 bases of the AGP, and the remaining bases from 67–90 were from the GP (Fig. 5, Mutant 11). When analyzed in the vacT7 system, Mutant 11 was able to replicate to levels that were ~86% that of the WT AGP (Fig. 5B). Thus, transfer of the AGP CRI and Region III in place of the corresponding sequences in the GP leads to an increase in RNA replication to ~40% of the AGP control, but this can be further in-

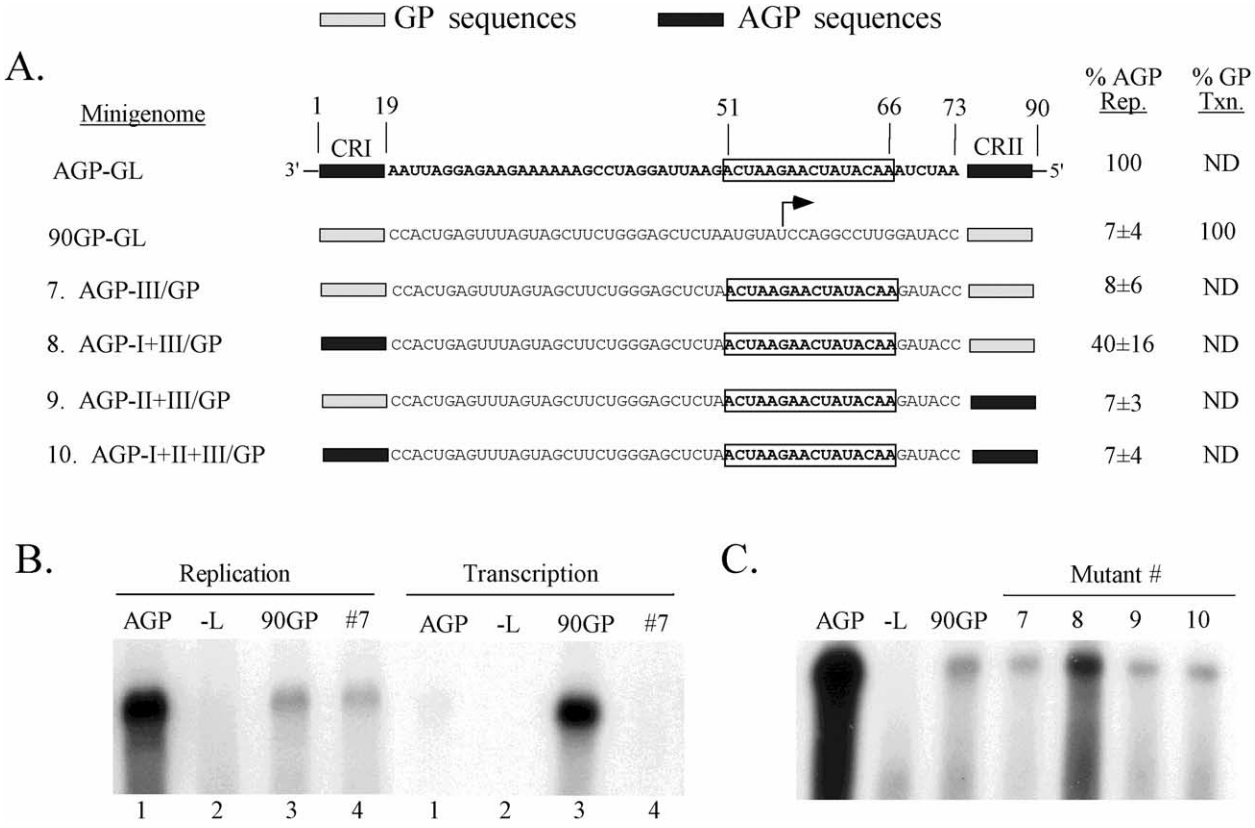

Fig. 4. Addition of AGP Region III and CRI to the GP results in a loss in transcription, and an increase in RNA replication. (A) Structures of WT and chimeric minigenomes. The sequence for the intervening region is shown for each minigenome with CRI and CRII represented as a black box for the AGP and a stippled box for GP. Region III is boxed in the AGP and the transcription start site is represented by an arrow in the GP. Mutant 7 contains Region III of the AGP replacing bases 51–66 of the GP. Mutants 8–10 contain Region III, along with CRI, CRII, or CRI+II sequences derived from the AGP, respectively. Replication levels are expressed as percentages of the levels determined for the WT AGP minigenome (average ± SD). The transcription level of Mutant 7 is expressed as a percentage of the level determined for the WT GP minigenome (90GP). ND, none detected. Representative Northern blots are shown in B and C.

creased to levels approaching that of the AGP when the promoter also contains the RNA segment between the AGP CRI and Region III.

One hypothesis to explain the enhanced replication of Mutant 8 is that substituting the AGP region between CRI and Region III added a positive-acting signal for replication to the GP. Alternatively, the WT GP could have a negative-acting signal that was removed by substituting with AGP bases 20–50. To distinguish between these possibilities, a minigenome was constructed such that CRI and Region III were derived from the AGP, but bases 20–50 between CRI and Region III were replaced with nonviral sequences (Fig. 5A, Mutant 12). Mutant 12 was found to direct RNA replication to ~81% that of the WT AGP minigenome (Fig. 5B). This result indicates that enhanced replication of chimeric minigenome 11 is due to removal of a negative-acting signal between bases 20 and 50 of the GP rather than addition of a positive-acting signal from the AGP. The effect of nonviral sequences on GP replication was only seen in combination with the AGP CRI and Region III, as evidenced by the finding that no increase in GP RNA replication was observed when bases 20–50 were replaced with nonviral sequences in the context of an otherwise WT GP (data not shown).

To determine if the negative-acting signal between bases 20 and 50 is an extension of the CRI element or of Region III, two additional minigenomes were constructed (Fig. 5, Mutants 13 and 14). Both minigenomes contain the AGP CRI and Region III and bases 67–90 of the GP. Mutants 13 and 14 differ by having bases 20–35 or 36–50 replaced with nonviral sequences, respectively (Fig. 5A). When assayed in the vacT7 system, Mutants 13 and 14 showed no significant increase in RNA replication over that of the GP (Fig. 5A and B). These data indicate that bases 20–50 must be substituted to increase RNA replication to AGP levels.

Taken together, the results in Figs. 4 and 5 show that the AGP CRI and Region III function as positive-acting signals for RNA replication, since when transferred together they can confer a partial increase in replication activity to the GP (e.g., Mutant 8). In addition, bases 20–50 of the GP contain a negative-acting signal, since replacement of this segment with nonviral sequences increased RNA replication (e.g., Mutant 11), but only in the context of the AGP CRI and Region III. However, data presented here (Figs. 3 and 5;

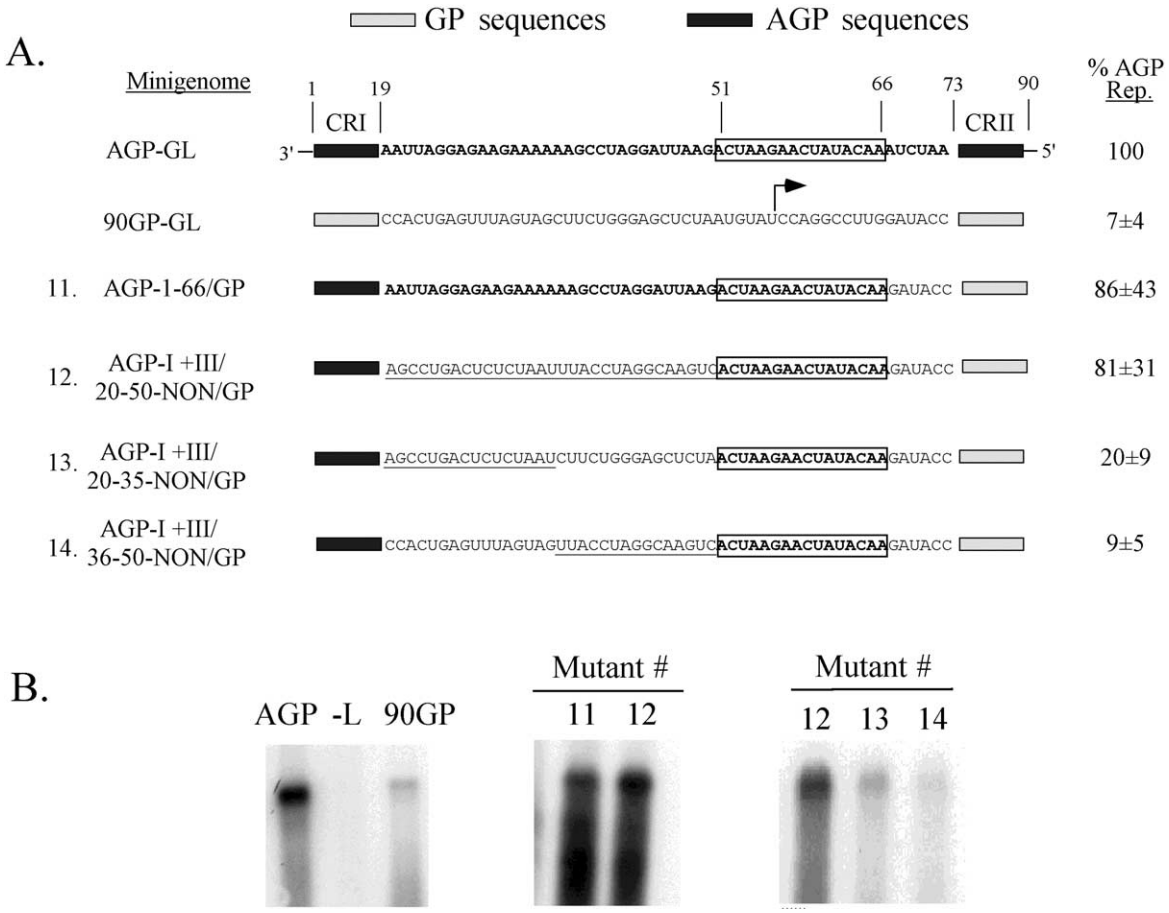

Fig. 5. Substitutions in the first 66 bases of the GP can lead to enhanced levels of RNA replication. (A) Structures of WT and chimeric minigenomes. The sequence for the intervening region between CRI and CRII is shown for each minigenome with the AGP and GP CRI and CRII shown as black or stippled boxes, respectively. Region III is boxed in the AGP and the transcription start site is represented by an arrow in the GP. Mutant 8 is the same as that shown in Fig. 5. Mutant 11 contains the first 66 bases of the AGP shown in bold, which include the AGP CRI and Region III, linked to bases 67–90 of the GP. Mutant 12 is the same as mutant 11 except that nonviral sequences (underlined) are substituted in place of bases 20–50. Mutants 13 and 14 are the same as Mutant 12, except that nonviral sequences (underlined) are only found from bases 20–35 (Mutant 13) or 36–50 (Mutant 14). Replication levels are expressed as percentages of the levels determined for the WT AGP minigenome (113-GL-AGP; average ± SD). Representative Northern blots of replication levels are shown in B.

Mutants 4, 7, and 11) indicate that the negative-acting sequence begins in CRI of the GP and extends to base 50. Thus, bases 1–66 of the AGP can confer higher replication to the GP by providing positive-acting signals (CRI and Region III) and removing a negative-acting signal.

Discussion

Current models suggest data that the relative strength of the GP and AGP is the major factor in determining differential levels of genomic and antigenomic RNA (reviewed in Lamb and Kolakofsky, 2001). Previous work with SeV and HPIV3 minigenomes has shown that the AGP directs higher levels of RNA replication than the GP and that mRNA transcription is the major synthetic event from the GP (Calain and Roux, 1993; Hoffman and Banerjee, 2000a; Taparrel and Roux, 1996). Our results indicate that the SV5 GP is also a weaker promoter for RNA replication, since the GP directs the synthesis of only ~7% of the level of RNA replication products seen with the AGP. The data presented here suggest that the low levels of RNA replication from the SV5 GP are due to lack of positive-acting signals present in the AGP and the presence of a negative-acting signal in the GP.

We have tested the hypothesis that substituting discrete promoter elements that are important for high-level RNA replication from the SV5 AGP alone or in combination in place of the corresponding GP regions would confer higher replication properties to the GP. Because the SV5 GP contains CRI and CRII elements but lacks Region III (Keller et al., 2001), an attractive possibility was that Region III alone could confer higher replication properties to the GP. While a GP-containing Region III substituted for bases 51–66 was not competent for transcription, the levels of RNA replication from the chimeric promoter were not increased above

WT GP levels. This result is consistent with previous results with SeV and RSV chimeric promoters, where it has been shown that transfer of increasing amounts of AGP sequence to the GP increased replication efficiency, but this increase did not strictly correlate with a decrease in transcription efficiency (Calain and Roux, 1995; Fearns et al., 2000). Taken together, these results support the proposal that the AGP Region III is not the sole determinant of differences in AGP and GP replication efficiency, and that the GP cannot be converted to a stronger promoter for replication by simply eliminating transcription.

The SV5 GP contains CRI and CRII elements that are similar but not identical in sequence to those found in the AGP (Fig. 1), suggesting that exchanges of CRI and CRII between the promoters would not have a significant effect on replication. CRII alone could be exchanged between promoters without significantly altering the level of RNA replication (Fig. 3). By contrast, exchanging CRI between the two promoters led to reduction in replication levels from any of the chimeric promoters (Fig. 3, Mutants 1, 3, 4 and 6). It was unexpected that replacing the GP CRI with the AGP CRI resulted in decreases in RNA replication since we hypothesized that any transferring AGP sequence would result in increases in RNA replication from the chimeric promoters. Taken together, this indicates that the ability of CRI to effect RNA replication is not only dependent on the primary sequence of CRI, but also the context in which the CRI element is recognized. In other words, the AGP CRI can function optimally with AGP sequence, whereas AGP CRI with GP sequences decreases RNA replication (Figs. 3 and 4). Our results demonstrate that a chimeric promoter containing CRI + Region III together showed a reproducible enhancement of RNA replication to approximately 40% that seen with the AGP (Fig. 5, Mutant 8). Thus, the AGP CRI, or CRI+CRII, decreases RNA replication when transferred to the GP alone, but CRI + Region III of the AGP can function together to partially increase RNA replication from the GP. Region III may act to enhance RNA replication from the AGP by interacting with the AGP CRI. Conversely, Region III could not exert this positive effect when combined with the GP CRI (Fig. 3, Mutant 4; Fig. 4, Mutants 7 and 9) Specifically, the three bases that differ between CRI of the GP and AGP are important for RNA replication from each promoter, possibly by interacting with the intervening sequence of either promoter in the correct context. This is consistent with results from extensive mutagenesis of the RSV and HPIV3 promoters in which changes in certain nucleotides within the 3′ terminal GP altered minigenome replication (Fearns et al., 2000; Hoffman and Bannerjee, 2000a). We propose that the SV5 GP CRI sequences contain a negative-acting signal, since transfer to the AGP significantly reduces RNA replication. For reasons that are presently unclear, a minigenome containing CRI, CRII, and Region III in the GP did not result in increased RNA replication and resembled a WT GP. This inclusion of CRII together with CRI and Region III from the AGP reduced the enhancement seen when CRI + Region III was substituted into the GP (Fig. 5, Mutant 8). Because exchanges of CRII between the AGP and GP had no effects in any other context (Fig. 3, Mutants 2 and 5; Fig. 4, Mutant 9), we hypothesize that this chimera may be defective for an unusual reason, such as producing a nascent RNA that is not properly encapsideated because of incorrect folding (see below).

We have found that the GP can direct RNA synthesis to levels approaching that of the AGP when the 3′ terminal 66 bases are derived from the AGP (Fig. 5). This transferred region includes that AGP CRI, Region III, and the segment between CRI and Region III from bases 20–50. Mutant 5 in Fig. 3 shows that RNA replication is significantly decreased when the AGP CRI is replaced with CRI from the GP and essentially converts the AGP to a GP. This led us to believe that a negative-acting signal for replication is present in the GP that begins in CRI and extends into the intervening region between CRI and CRII of the GP.

A key result from our study was the finding that high replication can also be achieved by substituting bases 20–50 of the GP with nonviral sequences, but only in the context of the AGP CRI and Region III. We have previously shown that substitution of a similar nonviral sequence into the corresponding segment of the AGP did not affect RNA replication levels (Murphy et al., 1998). We conclude from these results that the SV5 GP contains an element beginning in the GP CRI and spans bases 20–50 that has a negative effect on RNA replication, since replacement with nonviral sequences, of bases 20–50 in the context of the AGP CRI and Region III, enhances replication. Although we have not carried out extensive mutational analysis of the negative-acting sequence, RNA replication could not be enhanced by substituting nonviral sequence into either the 3′ or the 5′ half of the 20–50 base segment. For SeV, it has previously been shown that GP replication efficiency was increased by substitutions in GP bases 24–55 (Calain and Roux, 1993), and this corresponds well with bases 20–50 located between the SV5 CRI and Region III. Thus, the presence of a negative-acting element for RNA replication may be a common feature of the GP for other paramyxoviruses.

Our original hypothesis was that positive-acting signals from the AGP could be transferred to the GP as discreet elements to increase RNA replication from the GP. Our results are inconsistent with this straightforward hypothesis and instead suggest a more complicated model in which the level of RNA replication from the SV5 promoters is defined by a combination of negative- and positive-acting sequences within the first 66 bases of the genome and antigenome. This model is supported by two lines of evidence. First, transfer of the AGP CRI and Region III element in combination confer a partial enhancement of replication to the GP. We propose that these two AGP elements contain positive-acting sequences that promote RNA replication, since our previous work has shown that replacement of these regions in the AGP with nonviral sequences decreases replication

(Keller et al., 2001; Murphy et al., 1998). Second, we propose that the GP contains a negative-acting sequence that inhibits RNA replication that begins in CRI of the GP and extends to the location of Region III in the chimeric promoters (Figs. 4 and 5, Mutants 7–14). Together, it is only the combination of positive-acting CRI and Region III coupled with the removal of inhibitory sequences that can account for the differences in RNA replication directed by the GP and AGP.

Current models for paramyxovirus RNA replication involve signals that are located on both the nucleocapsid template and the nascent chain RNA that emerges from the viral polymerase and must be encapsidated by NP (Gubbay et al., 2001; Lamb and Kolakofsky, 2001). Based on the structure of the SeV nucleocapsid derived from electron micrographs (Egelman et al., 1989), we have previously proposed a model for the structure of the SV5 AGP that predicts that CRI and CRII are aligned on the same face of the helical nucleocapsid (Murphy et al., 1998). We have proposed that CRI and CRII are discontinuous elements brought together through turns of the nucleocapsid helix to serve as a binding site to position the viral polymerase at the ultimate 3′ end of the template (Murphy et al., 1998; Murphy and Parks, 1999). Although recent imaging studies suggest that SV5 differs from SeV in the number of NP subunits per turn of the helical nucleocapsid (Bhella et al., 2002), our proposed role for the SV5 CRI and CRII on the template is consistent with our finding that RNA replication from the AGP is very sensitive to small changes in the spacing of CRI and CRII (Murphy et al., 1998). In our model, Region III and the negative-acting segment contained in bases 20–50 of the GP map to the opposite face of the helix (Keller et al., 2001), suggesting that these segments function in RNA replication at a step other than on the template.

Encapsidation of newly synthesized viral RNA by NP is an essential step in paramyxovirus RNA replication (Lamb and Kolokofsky, 2001) and may function to increase the processivity of the viral polymerase (Gubbay et al., 2001; Vidal and Kolokofsky, 1989). Thus, a promoter element that enhances RNA replication could function as a signal on the nascent RNA strand to promote encapsidation by the NP protein as the RNA emerges from the viral polymerase. Thus, the negative-acting GP signals in CRI and bases 20–50 could function to decrease encapsidation of the nascent RNA strand by creating an RNA secondary structure that is not well recognized by NP. This would result in low levels of replication and predominantly transcription from the GP, which we have observed in minigenome assays (Fig. 2). In our model, CRII functions along with CRI on the template to align the viral polymerase for initiation at the 3′ end of the RNA, but the essential CRII element is not itself a determinant of high-level RNA replication. By contrast, our results support a model whereby the level of RNA replication is determined by the first 66 bases of SV5 RNA. Together, the AGP CRI and Region III, or the negative-acting signal in the GP, function as part of a nascent RNA chain to modulate replication at the level of encapsidation.

Sequences located at the ends of the paramyxovirus RNA genome can play important roles in the viral life cycle that extend beyond the control of RNA synthesis and packaging of viral RNA into progeny virions. Striking examples of a role for the viral promoters in pathogenesis have been reported for recombinant SeV mutants containing exchanges of the GP with AGP sequences (Garcin et al., 1998) or in naturally occurring field isolates with changes in GP sequences (Fujii et al., 2002). Work is in progress to determine the effect of altering the ability of the GP or AGP to direct replication on the noncytopathic nature of WT SV5 infections.

Materials and methods

Cells and viruses

Monolayer cultures of A549 human lung cells were grown and infected with virus as previously described (Murphy and Parks, 1997). Recombinant vaccinia virus (vTF7.3) expressing the T7 RNA polymerase (Fuerst et al., 1986) was grown and titered in CV1 cells.

Construction of plasmids containing SV5 genomic or chimeric promoters

cDNAs encoding the minigenome analogs were flanked on the 5′ and 3′ ends by the T7 polymerase promoter and DNA encoding the hepatitis delta virus ribozyme, respectively (Murphy and Parks, 1997; Pattnaik et al., 1992). All minigenomes were constructed such that the product generated by the T7 polymerase would be genomic sense RNA containing a 113-base 5′ L-tr end and various 3′ end promoters derived from AGP, GP, or nonviral sequences. The minigenome containing the wild-type SV5 AGP is designated 113-GL-AGP, which 113 SV5-specific 5′-terminal nucleotides linked to the Green Lantern (GL; Gibco BRL) open reading frame followed by the terminal 90 bases of 3′ antigenomic RNA (corresponding to the SV5 AGP). The minigenome containing the wild-type GP of SV5 is designated 113-GL-90GP and is exactly the same as 113-GL-AGP, except that it contains the first 90 bases of 3′ genomic RNA. To construct 113-GL-90GP, a plasmid encoding a dicistronic internal deletion-type minigenome (pMF2; Rassa and Parks, 1999) was digested with *Eco*RI and *Asp*718 and used in a PCR along with SP6 primer and an internal primer that introduced a *Stu*I site adjacent to the 90-base leader-NP region. The resulting product was digested with *Stu*I and *Sph*I and inserted into the corresponding site in 113-GL-90B/Not-KO, a plasmid in which the *Not*I site normally found in the GL sequence has been deleted. Details of the mutagenesis procedures used to creat individual minigenomes are available on request from the

authors. Briefly, insertions, deletions, and chimeric promoters were generated using the megaprimer method (Parks, 1994) with an internal primer to introduce the mutation and SP6 primer. The resulting PCR products were digested with *Stu*I and *Sph*I and inserted into the corresponding site of 113-GL-AGP/Not-KO. The nucleotide sequence of all PCR-derived DNA segments was determined.

Analysis of in vivo RNA synthesis from cDNA-derived components

Dishes (3.5 cm) of A549 human lung cells were infected (multiplicity of infection ~5) for 1 h with vTF7.3 (Fuerst et al., 1986). These cells were subsequently cotransfected with the plasmid containing the minigenome of choice (1 μg) and three support plasmids: pGem3-L (2 μg), pGem3-P (0.2 μg), and pUC19-NP (3 μg), as described previously (Murphy and Parks, 1997), using Lipofectin reagent for Fig. 2. For Figs. 3–5, FuGene 6 reagent (Roche, Indianapolis, IN) was used in place of Lipofectin for improved transfection efficiency. Control plasmid (pGem3) was used to normalize the amount of transfected DNA. RNA was harvested from two 3.5-cm dishes using Trizol reagent at ~40–48 h post-transfection. To analyze RNA replication, cell lysates were treated with micrococcal nuclease prior to Trizol extraction as described (Peeples and Collins, 2000; Keller et al., 2001). To analyze transcription products, purified RNA was selected using oligo(dT) (Rassa and Parks, 1999). All RNAs were analyzed by Northern blotting as described previously.

A ^{32}P-labeled minus-sense GL-specific riboprobe (Murphy et al., 1998) was generated from *Bam*HI-linearized pGem2-GL by SP6 RNA polymerase in the presence of [^{32}P]CTP. Membranes were hybridized with purified riboprobes for 1 h at 70°C in ExpressHyb (Clontech, Palo Alto, CA). The membranes were then washed three times for 15 min with 2× SSC/0.1% SDS, 0.2× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS, respectively. RNA levels were quantitated using PhosphorImager analysis and are expressed as percentages of the 113-GL-AGP or 113-GL-GP wild-type levels.

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